

Deregulation of microRNA Processing in Idiopathic Pulmonary Fibrosis

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Introduction

The Disease: Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is a devastating interstitial lung disease with an incidence in the United States of 42.7 to 63 per 100,000 and an incidence in Europe of 16.3 to 17.4 per 100,000 (Nalysnyk *et al.* 2012). Incidence and severity of IPF increases with age, but on average projected survival is just 3-4 years post-diagnosis (Raghu *et al.* 2014). Until recently, treatment options have been limited and mostly palliative, with little impact on survival. For many years, the only established treatment shown to extend life was lung transplantation. However, lungs are the most difficult organs to transplant, with patients surviving an average of just 4.6 years post-transplantation (NIH 2014). Just recently, two drugs (pirfenidone and nintedanib) have been approved for treatment of IPF. However, even with such treatments the unyielding progression of IPF is only slowed, and a more effective treatment for IPF remains both necessary and illusive (as reviewed in Jenkins and Goodwin 2014).

Fibrosis occurs in organs other than the lungs, including the liver, heart and kidney (He *et al.* 2013). The process is defined by excess connective tissue that spreads throughout the affected organ, often leading to organ failure (Ahluwalia *et al.* 2014). This scarring is caused by abnormal proliferation of fibroblasts and accumulation of extracellular matrix (ECM) proteins (Jenkins and Goodwin, 2014). Fibroblasts are cells that monitor signals from the ECM and continuously remodel the ECM by secreting and rearranging ECM proteins. Fibrosis is a tightly regulated process that occurs normally during wound healing and ceases once the wound is healed. The progressive scarring characteristic of IPF results from deregulation of the wound healing response, leading to damage that reduces the ability of the lungs to exchange oxygen and ultimately results in respiratory failure (Jenkins and Goodwin 2014, King Jr *et al.* 2011).

Origins of IPF

The pathogenesis of IPF is not fully understood, but the predominant theory in the field is that fibrosis results from an atypical wound healing response to alveolar epithelial cell injury (Jenkins and Goodwin 2014). This includes injuries associated with day-to-day hazards of living, including inhalation of dust, aspiration of stomach acid, and viral infection. Injury activates a physiological inflammatory response in the lungs, which in turn activates resident fibroblasts causing them to divide and secrete ECM proteins. In normal lung repair, the inflammatory pathway is shut off when the wound is healed. However, the atypical response observed in IPF occurs when the inflammatory response becomes deregulated, resulting in continuous activation of fibroblasts and deposition of ECM proteins (reviewed in Ahluwalia *et al.* 2014 and Jenkins and Goodwin 2014).

The mechanism by which deregulation of the wound healing pathway occurs remains unclear; however, it is likely that genetic susceptibility plays a role similar to the one it plays in cancer. Familial IPF accounts for only about 20% of all reported cases of IPF, but many sporadic cases of IPF have been attributed to mutations in genes involved with host defense, intercellular adhesion and DNA repair. Both familial and sporadic IPF have been associated with mutations in tumor suppressor genes, indicating that similar factors contribute to the initiation of fibrosis and cancer. Other parallels between the fibrosis and cancer are abnormal cell proliferation and global suppression of microRNAs involved in cell cycle control. Unlike cancer, fibrosis is not metastatic in nature and does not arise from clonal origins (Jenkins and Goodwin 2014).

Role of the Extracellular Matrix in IPF

The ECM is composed of proteins and carbohydrates secreted and continuously rearranged by fibroblasts (Cox and Erler, 2011). The ECM of the human lung is composed of a variety of proteins, including collagen I protein, responsible for tensile strength, as well as elastins, laminins and collagen IV (Booth *et al.* 2012). These proteins serve as a structural scaffold on which cells grow and proliferate, and it is their distribution and arrangement that determines the macroscopic and microscopic structure of organs. Although the ECM was originally thought to be an inert scaffold, it is now being studied as a major regulator of cellular function (reviewed in Cox and Erler, 2011). These studies have demonstrated an active role for the ECM in wound healing as well as in cancer and fibrosis.

In IPF, the “stiffness” of the ECM increases due to excessive deposition of collagen I and other ECM proteins. Relative stiffness is measured using an indicator of elasticity, the mean shear modulus. Physiological lung ECM is compliant, with a median shear modulus of approximately 0.5 kilopascals (kPa). In contrast, IPF ECM is relatively stiff, with a median shear modulus of approximately 3.0 kPa, with some areas of excess collagen that measure >15 kPa (Liu *et al.* 2010). This stiffness, defined as the ECM’s ability to resist mechanical deformation, has been shown to promote lung fibrosis through the regulation of microRNAs (Huang *et al.* 2012, Parker *et al.* 2014).

One possible mechanism for microRNA regulation is through mechanotransduction pathways. Mechanotransduction is a process by which cells can respond to mechanical stimuli (i.e. changes in the environment) by transmitting a biochemical signal (Huang *et al.* 2012). In IPF, increased stiffness of the ECM serves as one such mechanical signal, resulting in differentiation of fibroblasts into myofibroblasts. Myofibroblasts are fibroblasts that exhibit

increased motility and ECM reconstitution after injury, and are crucial for initiation and progression of fibrosis. A recently published study demonstrates that the activity of transient receptor potential vanilloid 4 (TRPV4), a calcium-initiated cation channel, is upregulated in IPF-derived lung fibroblasts and that TRPV4 knockout prevents fibrosis in mice (Rahaman *et al.* 2014). In addition, this study also shows that TRPV4-dependent calcium influx is responsible for transmitting the mechanical signal created by fibrotic stiffness and is necessary for myofibroblast differentiation (Rahaman *et al.* 2014). These findings provide evidence for the role of mechanotransduction in the initiation of fibrosis; however, the exact mechanism and signaling pathway of microRNA regulation by ECM has yet to be thoroughly investigated.

Role of MicroRNA-29 in IPF

MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression that act by binding to the 3' untranslated regions of target mRNAs, blocking translation and promoting mRNA degradation (He *et al.* 2013, van Kouwenhove *et al.* 2011, Mori *et al.* 2014). Suppression of microRNA-29 (miR-29) has been implicated in the initiation and progression of fibrosis in the heart, liver, kidney, and lung (He *et al.* 2013). miR-29 consists of a microRNA family of four different transcripts, miR-29a, -29b-1, -29b-2, and -29c. These are transcribed in pairs, with miR-29a and -29b-1 located on 7q32.3 and miR-29c and -29b-2 located on 1q32.2. miR-29 transcripts are initially polycistronic and are later cleaved into their separate mature forms during microRNA processing (Mott *et al.* 2010). The two forms of miR-29b are genetically identical, distinguishable only by their chromosomal location and transcriptional pairing.

miR-29 is negatively regulated by TGF- β /Smad signaling, which has been shown to play a role in fibrosis. Suppression of miR-29 in turn relieves suppression of fibrosis-affiliated genes, including collagen I, collagen III, and fibronectin (Xiao *et al.* 2012). Experiments in Smad3

knockout mice demonstrate that miR-29 overexpression can prevent and halt progression of bleomycin-induced fibrosis (Xiao *et al.* 2012). This data indicates that suppression of miR-29 in fibroblasts may be at least one of the elements responsible for the increased protein production observed in IPF. In order to study how a pathological ECM contributes to IPF pathogenesis, our group decellularized ECM from control and IPF lung tissue. Results from these experiments indicate that IPF ECM in turn suppresses miR-29 expression, establishing a positive feedback loop (see Figure 1). This mechanism of positive feedback is a potential factor in the progression of fibrosis in IPF (Parker *et al.* 2014).

MicroRNA Processing

As reviewed in He *et al.* (2013) and Goodall *et al.* (2013), microRNA processing is established as a canonical and highly conserved pathway by which miRNAs are transcribed in the nucleus from non-coding regions of DNA, processed, and exported to the cytosol where they bind and suppress expression of target mRNA. miRNAs are first transcribed by RNA polymerase II as primary miRNAs (pri-miRNAs), which are then capped, spliced and polyadenylated. Pri-miRNAs spontaneously form hairpin structures, which are recognized by the microprocessor complex consisting of Drosha, an RNase III endonuclease, and its binding partner DiGeorge syndrome critical region 8 (DGCR8). Drosha cleaves the double-stranded stem of the pri-miRNA, producing a precursor miRNA (pre-miRNA) that is 60-70 nucleotides long and retains its hairpin structure. Two accessory RNA-binding proteins, p68 and p72, are required for processing of pri-miRNA to pre-miRNA. Once cleaved, the pre-miRNA molecule is exported to the cytoplasm by exportin 5 where it encounters Dicer, another RNA III endonuclease. Dicer cleaves the pre-miRNA once more, producing a mature miRNA duplex. Next, an Argonaute protein (Ago) unwinds the duplex and the mature single-stranded miRNA is loaded onto the

RNA-induced silencing complex (RISC). The mature miRNA is capable of binding complementary target mRNA and suppresses expression either through translational repression or mRNA cleavage and degradation by RISC (see Figure 2).

The Hippo Pathway: Fibrosis as a Cancer Phenocopy

As more is discovered about both cancer and IPF, more similarities between the two diseases are surfacing. Both cancer and fibrosis exhibit global suppression of microRNA, and in cancer there is evidence that this suppression is due to deregulation of the Hippo pathway. Cancer cells lose the ability to sense and respond to cell density, resulting in suppression of miRNA and an increase in expression of target genes that are involved in cell cycle and cell division. This results in increased proliferation leading to tumorigenesis (Mori *et al.* 2014).

The Hippo signaling pathway is a tumor-suppressor pathway that regulates microRNA processing in a cell-density dependent manner. Physiologically, Hippo signaling helps a cell respond to its environment. When cell density is low, the Hippo pathway is off and unphosphorylated YAP is able to translocate to the nucleus. Nuclear YAP functions as a transcriptional co-activator that regulates expression of target genes associated with increased proliferation and oncogenic transformation (Zhao *et al.* 2008). However, when cell density is high, the Hippo pathway is turned on and phosphorylated YAP is prevented from translocating to the nucleus, resulting in suppression of genes involved in proliferation. Additionally, the localization of YAP is important to microRNA processing because nuclear YAP is able to bind to p72, the aforementioned accessory binding protein required for microRNA processing (van Kouwenhove *et al.* 2011). Sequestration of p72 in the nucleus by YAP effectively blocks microRNA processing, resulting in an accumulation of pri-miRNA transcripts in the nucleus and suppression of mature miRNA in the cytoplasm (see Figure 3) (Mori *et al.* 2014).

Although the processes leading to miRNA suppression in fibrosis are not as completely understood, it is possible that the Hippo pathway is also involved in initiation and progression of fibrosis. The role of the Hippo pathway in fibrosis is implicated by a study demonstrating that overexpression of YAP is sufficient to produce fibrosis both in cell culture and in a mouse model (Liu *et al.* 2014). Additionally, we are interested in overexpression of YAP because it is a direct target of miR-29 b-1/a (Tumaneng *et al.* 2012). Involvement of the Hippo pathway in fibrosis would help to explain the suppression of miR-29 that has been observed in fibroblasts cultured on decellularized IPF ECM tissue (Parker *et al.* 2014).

Specific Aims

- (1) Determine whether miR-29 suppression is a cell-inherent trait of fibroblasts isolated from IPF patients versus control patients.
- (2) Determine whether miR-29 suppression in IPF is due to inhibition of microRNA processing mediated through the Hippo pathway.

The purpose of this study is to determine unknown players involved in the suppression of miR-29 observed in IPF. We hypothesize that IPF ECM influences miR-29 suppression by deregulating the Hippo pathway in a manner analogous to what has been shown in cancer.

Materials & Methods

Primary human lung fibroblast cell lines: Cell lines from six donors were isolated from human lung tissue obtained from the University of Minnesota's Tissue Procurement Facility using tissue explant methods to isolate fibroblasts. These consist of three control samples derived from histologically uninvolved tissue adjacent to a resected tumor and three IPF samples derived from regions of the lung that met criteria for interstitial pneumonitis. Patient-derived material was de-identified and approved for use by University of Minnesota's Institutional Review Board. Tissue was minced and cultivated on 35-mm tissue culture plastic in explant medium (DMEM with 20% FBS and 200 IU/mL Streptomycin, 200 IU/mL Penicillin) at 37°C, 95% air/5% CO₂ for 2-3 weeks until confluent. Each cell line was released from the 35-mm plate using trypsin-EDTA and allowed to expand in a 150-mm tissue culture dish (considered passage 1) in fibroblast growth media (low glucose DMEM, 10% FBS, 100 IU/mL Streptomycin, 100 IU/mL Penicillin). Fibroblasts were characterized by their spindle-like morphology, expression of vimentin and alpha-smooth muscle actin, and lack of expression of factor VIII and surfactant C (as described in Parker *et al.* 2014). Cells were sub-cultivated at a 1:3 split ratio (37°C, 5% CO₂), then frozen in 50% fibroblast growth media and 50% freezing media [(20% dimethylsulfoxide (DMSO) in fetal bovine serum (FBS))]. All experiments were conducted using freshly activated cell lines between passages 4 and 7.

Decellularized human lung ECM: Human lung tissue (obtained as described in the previous section) was cut into 30 cm³ pieces and frozen at -70°C. Frozen tissue adhered to a plate was equilibrated to -15°C, cut into 400 µm slices, and frozen (-20°C) in tissue culture dishes containing PBS. Tissue was thawed as needed for decellularization and cut into 1 cm² pieces for use. Lung slices were decellularized by 3 overnight wash steps of 1% SDS, 1% Triton-X100, and

NaCl, lysing any cells remaining on ECM and rinsing away cell remnants in decanted washes (as described in Parker *et al.* 2014). The decellularized ECM that remained was rinsed in PBS and 1 piece from each patient sample was placed in a 15 mL conical tube with 2 mL antibiotics (200 IU/mL Streptomycin, 200 IU/mL Penicillin) in PBS and kept at room temperature until ready for use (up to 2 hours). Each 15 mL conical tube contained three control or three IPF decellularized ECM sections, one from each patient sample. Cells (obtained as described in previous section) were released from cell culture dishes using trypsin-EDTA and concentration determined using a hemocytometer. PBS containing antibiotics was poured off of the ECM in 15mL conical tubes and replaced with 200,000 cells in 2 mL survival media (low glucose DMEM, 1% FBS, 100 IU/mL Streptomycin, 100 IU/mL Penicillin). Cells were grown for 18 hours oscillating at ~6-8 revolutions per minute (37°C, 5% CO₂/95% air).

RNA isolation, reverse transcription, and quantitative PCR analysis: After 18 hours of incubation, ECM slices containing cells were washed in PBS twice, blotted dry and placed in 1 mL Tri-Reagent (Sigma-Aldrich) and chloroform extracted. Isolated RNA was reverse transcribed using miScript II RT KIT (Qiagen) according to manufacturer's instructions, allowing us to measure mRNA, precursor microRNA, and mature microRNAs from the same sample. Because mature microRNA are only around 22 base pairs long and are thus too short to be identified using typical 18 base pair primers, the manufacturers developed a universal tag that is incorporated into a single round of reverse transcribed complementary DNA product. This allows the mature miRNAs to later be detected by a reverse primer complementary to the universal tag during qPCR (Qiagen). A primer specific to each mature microRNA sequence is also used to identify them from one another. Precursor microRNA were detected using primers that recognize the stem loop hairpin structure. All primers used for measuring mature and

precursor miRNA were proprietary, validated & confirmed by Qiagen (see Table 1 for a list of primers used). qPCR was performed using SYBR-Green PCR Kit (Qiagen) and analyzed using a Roche Light Cycler 1.5 (Software Version 3.5). Cycle number for each product was normalized to RNU (mature) or GAPDH (precursor) and statistics were conducted using a two-tailed student t-test.

Western blot: After 18 hours of incubation, ECM containing cells were washed in PBS twice, blotted dry and placed in 125 μ L of lysis buffer [150 mM NaCl, 1 mM EGTA, 50 mM Tris, pH 7.4, 1% Triton X-100, 1% Nonidet P-40, 1% sodium deoxycholate, protease inhibitors (Roche)]. Proteins were separated by size using gel electrophoresis on a 10% polyacrylamide gel and were then transferred to a nitrocellulose membrane before probing for proteins using specific antibodies. The membrane was incubated overnight with primary antibodies at 4°C: rb anti-YAP (cell signal 14074; 1:2000), rb anti-GAPDH (Santa Cruz Bio sc-25778; 1:2,000). After incubation with primary antibody, the blot was washed three times at room temperature in Tris-buffered saline with Tween 20 (TBS-T) buffer and incubated in secondary antibody [goat anti-rabbit IgG horseradish peroxidase (HRP) (Calbiochem 401393; 1:10,000)] at room temperature for 1 hour. Washes in TBS-T were repeated and the blot was developed using enhanced chemiluminescence (ECL) western blotting detection (GE Healthcare W9488333) following the manufacturer's protocol.

Results

To address the question of whether suppression of miR-29 is an inherent trait in cells derived from an IPF patient, levels of mature miR-29 were measured in control and IPF cells grown on control and IPF ECM. We chose miR-29a and -29c as readouts of processed microRNA as miR-29b is expressed polycistronically with both miR-29a and -29c. miR-214 was measured as an internal control as its levels should remain constant in cells grown on control and IPF ECM, and is a Hippo pathway non-responsive microRNA (Mori *et al.* 2014). RNU6 was used for normalization because it is a small nuclear RNA found in abundance that is widely used as an endogenous control. The results showed that mature miR-29a is suppressed in control fibroblasts grown on IPF ECM, but is not significantly decreased in IPF fibroblasts on IPF ECM. However, the results for IPF fibroblasts were trending towards significant decrease of miR-29a, with one outlying cell line demonstrating increased levels of miR-29a on IPF ECM. Mature miR-29c is suppressed in both IPF and control fibroblasts on IPF ECM (Figure 1A). These data suggest that regulation of miR-29a and -29c is not a cell-inherent trait of IPF fibroblasts.

To address whether suppression is occurring at the level of microRNA processing, precursor miRNA levels for miR-29a and miR-29c were measured, along with precursor miR-214. GAPDH, another relatively abundant housekeeping gene, was used as a normalization control. Precursor miR-29a accumulates only in IPF fibroblasts on IPF ECM, while precursor miR-29c does not significantly increase in IPF or control fibroblasts (Figure 1B). These results are surprising because suppression of mature forms doesn't correspond to accumulation of precursor forms. Additionally, miR-214, which was included as an unchanging internal control, was increased on IPF ECM in control fibroblasts. However, the data for individual cell lines trends towards significance for accumulation of precursor miR-29a and -29c, with one major

outlier in both IPF and control fibroblasts for precursor miR-29c that likely skewed the data. Additionally, there is an outlier in the data for precursor miR-214 that may help explain why the data shows it is increased in control fibroblasts on IPF ECM. These outliers in individual cell lines helps to explain the surprising results and indicate that microRNA processing is likely involved in the suppression of miR-29 observed in IPF despite disparities seen in the data.

The last question raised in this study was whether the Hippo pathway is involved in the inhibition of microRNA processing observed in IPF. To address this, a western blot was performed to determine if there is an accumulation of YAP in cells on IPF ECM. Five independent cell lines (2 IPF and 3 control) were used in this experiment. The results showed that there was no significant difference in total YAP levels between control fibroblasts on control and IPF ECM. The same was true for IPF fibroblasts (see Figure 5). Although this demonstrates that overall YAP levels are the same, it does not take into account the subcellular localization of YAP and what impact phosphorylation may have on YAP's ability to affect microRNA processing.

Discussion

Although the downstream effects of miR-29 suppression in fibrosis have been previously examined, we still do not understand the mechanism of microRNA suppression in fibrosis. This study attempts to address this gap by answering two questions: (1) is miR-29 suppression is a cell-inherent trait of IPF fibroblasts and (2) is miR-29 suppression due to inhibition of microRNA processing caused by deregulation of the Hippo tumor suppressor pathway. To address these questions, we seeded control and IPF fibroblasts on decellularized control and IPF ECM and measured the expression of mature and precursor miR-29a and -29c as well as YAP protein expression. The results indicate that miR-29 suppression is not a cell-inherent trait of IPF fibroblasts and that microRNA processing is inhibited in fibroblasts on IPF ECM (see Figure 4B).

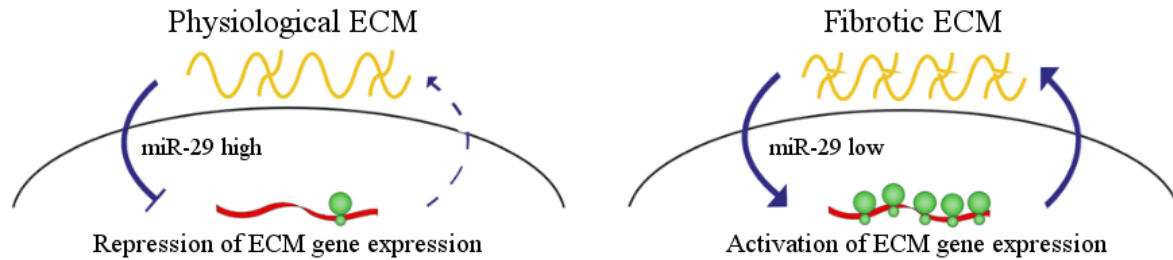
Deregulation of the Hippo pathway has been shown to be responsible for miRNA suppression and consequent loss of cell density-dependence in cancer (Mori *et al.* 2014). In this study, we hypothesized that deregulation of the Hippo pathway is also responsible for microRNA suppression in IPF. From the data, we conclude that inhibition of microRNA processing plays a role in miR-29 suppression in cells on IPF ECM, although not to the exclusion of transcriptional regulation. It remains unclear, however, if microRNA suppression is due to changes in regulation of the Hippo pathway. If this were the case, we would expect to see an accumulation of YAP in cells on IPF ECM due to loss of cell density dependence. Our data shows no change in overall YAP expression, but does not rule out involvement of the Hippo pathway in fibrosis (see Figure 5). Thus, further study would be necessary to elucidate the role of the Hippo pathway in miR-29 suppression in fibrosis. One possibility would be to investigate the phosphorylation state and localization of YAP to further examine the Hippo pathway as a regulator of miR-29 expression

in IPF. Additionally, TAZ, YAP's co-activation partner, could also be investigated as a player in the regulation of microRNA processing.

If deregulation of the Hippo pathway is not the source of microRNA processing inhibition in IPF, it is possible that other downstream regulators of microRNA processing are affected. One likely candidate for this is the microprocessor complex, consisting of Drosha and DGCR8 as well as p72 and p68, which is responsible for cleaving pri-miRNA to pre-miRNA. Another potential candidate is Dicer, which is located in the cytoplasm and cleaves pre-miRNA to its mature form. Additionally, there has been some research published implicating down-regulation of Argonaute proteins in IPF. Argonaute proteins are catalytic subunits that function further downstream in microRNA processing, unwinding the microRNA duplex and enabling the mature single-stranded microRNA to associate with RISC and thus bind to its target mRNA (see Figure 2). Although research has demonstrated Argonaute proteins suppression in IPF, it is not clear whether this is due to inhibition at the transcriptional level or to post-transcriptional modifications affecting stability and/or activity (Oak *et al.* 2011). Thus, further research investigating the mechanisms behind Argonaute suppression in IPF may further elucidate the role of microRNA processing inhibition in miR-29 suppression.

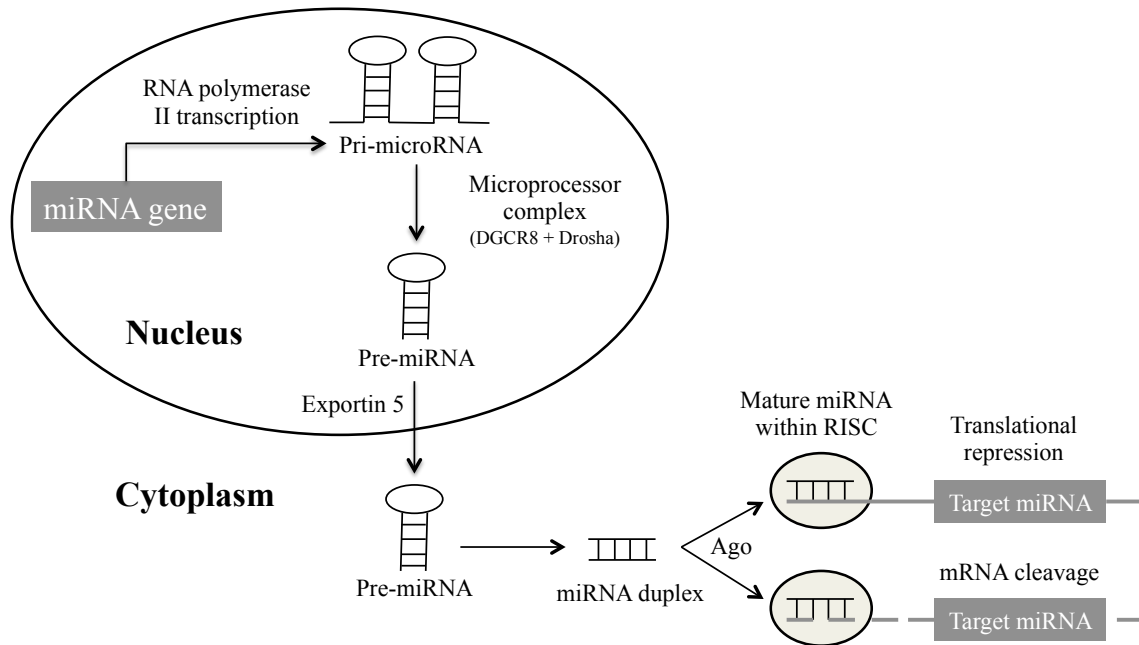
Although the data presented here do not support our hypothesis that miR-29 suppression is due to deregulation of the Hippo tumor suppressor pathway, the results do support our hypothesis that suppression is due to inhibition of microRNA processing. Repeating these experiments with additional cell lines may yield clearer results from a statistical standpoint. Additionally, future studies can look at other components of microRNA processing in order to determine the upstream regulators of miR-29 suppression in IPF. This information would lead to a better understanding of the disease and better potential targets for treatment of IPF.

Figures & Tables



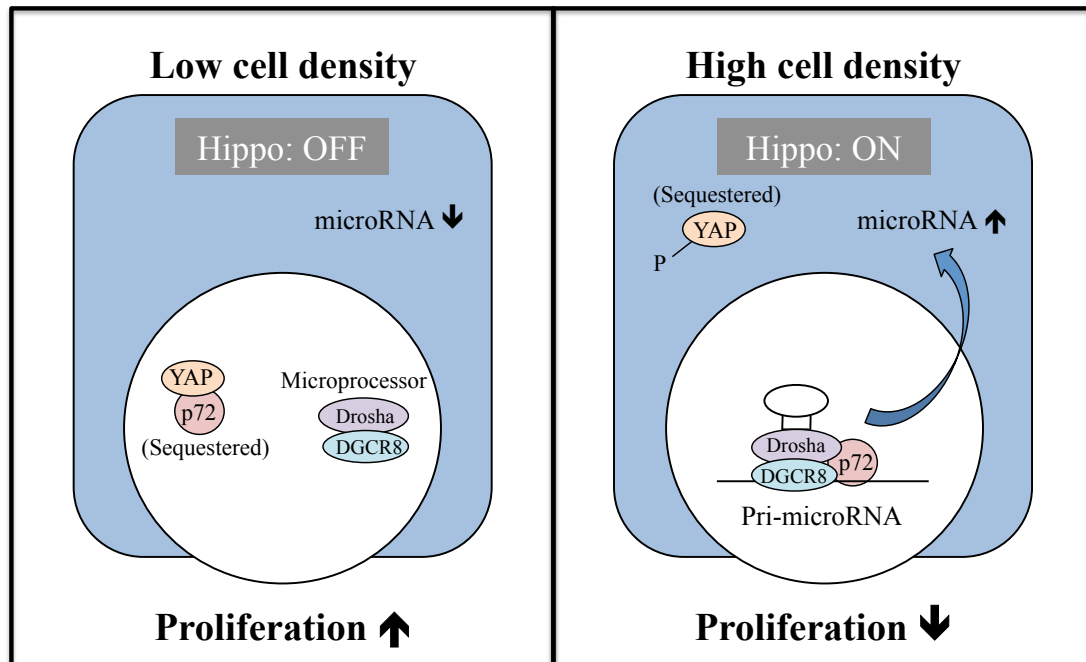
Adapted from Parker *et al.* (2014). *J. Clin. Invest.*

Figure 1: Positive feedback loop between microRNA-29 and the extracellular matrix. This figure demonstrates the interaction between miR-29 transcripts and ECM gene expression. (L) Increased miR-29 expression represses expression of ECM genes, resulting in physiological ECM. (R) Decreased miR-29 expression allows activation of ECM genes, resulting in increased protein deposition. This leads to ECM stiffening, greater suppression of miR-29, and further activation of ECM gene expression, resulting in a positive feedback loop and spread of fibrosis.



Adapted from Goodall *et al.* (2013) *Front Cell Neurosci.*

Figure 2: MicroRNA Processing. Pri-miRNA (the primary microRNA transcript) is transcribed from the miRNA gene by RNA polymerase II, cleaved to precursor-miRNA (pre-miRNA) by the microprocessor complex and exported to the cytoplasm by exportin 5. In the cytoplasm, Dicer cleaves pre-miRNA to a mature miRNA duplex, which is then unwound by an Argonaute protein (Ago) and the single-stranded mature miRNA (complementary to target) is loaded onto an RNA-induced silencing complex (RISC). Together, RISC and the miRNA bind to its target mRNA, repressing translation and/or inducing mRNA cleavage and consequent degradation.



Adapted from Mori *et al.* (2014.) *Cell*.

Figure 3: Hippo signaling regulation of microRNA expression. Yes-associated protein (YAP), a downstream target of the Hippo signaling pathway, plays a role in regulation of microRNA processing. (L) At physiological low cell density, the Hippo pathway is off. YAP is unphosphorylated and is able to translocate to the nucleus where it sequesters the accessory binding protein p72, effectively preventing microRNA processing. This leads to an accumulation of nonfunctional pri-miRNA and a decrease in the amount of mature miRNA, resulting in increased expression of genes involved in proliferation. (R) At physiological high cell density the Hippo pathway is on. YAP is phosphorylated and sequestered in the cytoplasm, allowing p72 to associate with the microprocessor complex and progression of microRNA processing, demonstrated by an increase in levels of mature microRNA. This results in a decrease in proliferation (contact inhibition).

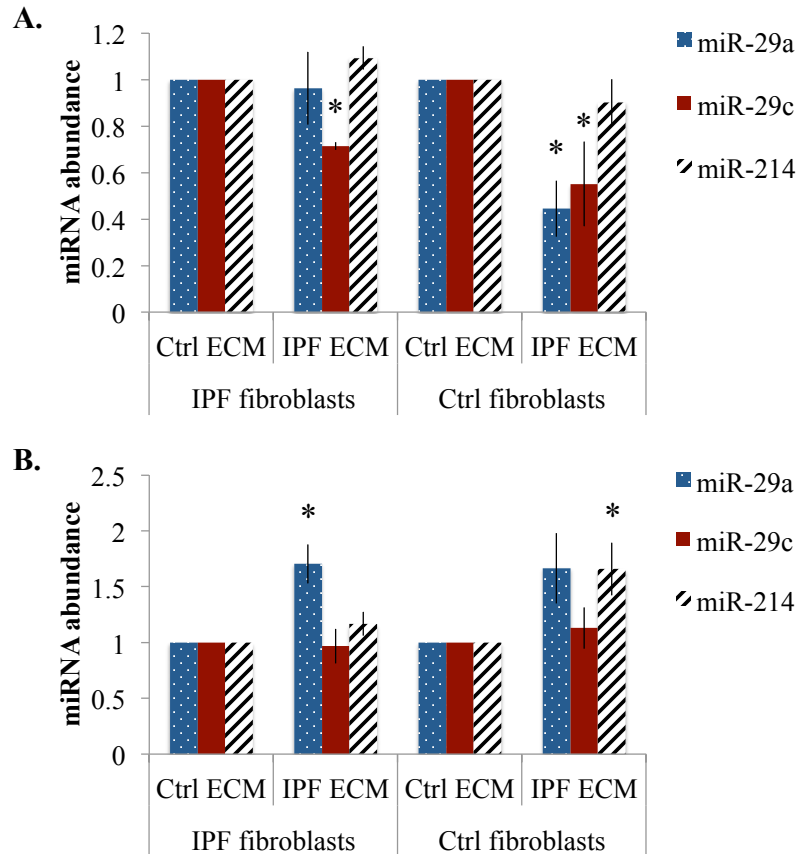


Figure 4: IPF ECM suppresses mature miR-29 and increases precursor miR-29

independent of cell type (Averages). 3 control lung fibroblast cell lines and 3 IPF lung

fibroblast cell lines were cultured on control or IPF ECM for 18 hours and qPCR performed. The averages of 3 cell lines are shown, with error bars representing standard error. *P < 0.05 versus

cells on control ECM, calculated using a two-tailed student t-test. (A) Mature miR-29a, -29c, and -214 normalized to RNU6. (B) Precursor miR-29a, -29c, and -214 normalized to GAPDH.

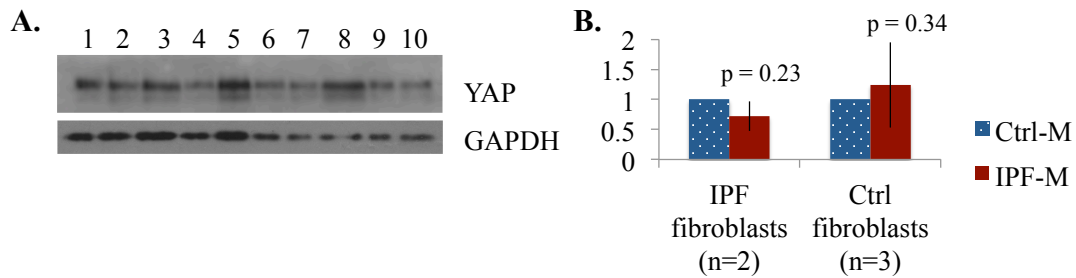


Figure 5: ECM does not regulate YAP abundance. (L) 3 control lung fibroblast cell lines and 2 IPF lung fibroblast cell lines were cultured on control or IPF ECM for 18 hours. Total cell lysates were used for immunoblot probing for YAP and GAPDH. Statistics were performed using a two-tailed paired t-test on data normalized to GAPDH and YAP levels in cells on control ECM. (Lanes 1-4: Two individual IPF cell lines on control ECM (lanes 1,3) and IPF ECM (2,4). Lanes 5-10: Three individual control cell lines on control ECM (5,7,9) and IPF ECM (6,8,10). P values calculated using a paired two-tailed student t-test.

Table 1: Qiagen primers for qPCR analysis of precursor and mature microRNA probes

| Precursor | Probe sequence data | Mature | Probe sequence data |
|-----------|---------------------|---------|---------------------|
| miR-29b/a | MP00001736 | miR-29a | MS0003262 |
| miR-29b/c | MP00001757 | miR-29c | MS00003269 |
| miR-214 | MP00001540 | miR-214 | MS00009093 |
| GAPDH | QT00079247 | RNU6 | MS00033740 |
| RRN18S | QT00199367 | - | - |

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